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Time dependent production of cytokines and eicosanoids by human monocytic leukaemia U937 cells; effects of glucocorticosteroids

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In the present study the human monoblast cell line U937 has been used as a model to study the function of human mononuclear phagocytes in asthma. The kinetics of the production of eicosanoids and cytokines, which are thought to play a role in the pathogenesis of asthma, were studied. In addition, the effects of glucocorticosteroids were investigated, as these drugs are of great importance for the treatment of asthmatic patients. After stimulation with phorbol-12 myristate acetate (PMA) for 24 h, U937 cells were cultured in the absence or presence of lipopolysaccharide (LPS: 1 and 5 $\mu g\ ml^{-1}$) and glucocorticosteroids (budesonide, fluticasone propionate and prednisolone: 10^{-11}, 10^{-9} and 10^{-7} M) for 96 h. The production of interleukin- 1β (IL-1β), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and thromboxane B_2 (TxB_2) gradually increased in time after stimulation with LPS, whereas the transient production of tumor necrosis factor alpha (TNF-α) reached its maximum between 6 and 12 h. Interferon-gamma (IFN-γ), interleukin-10 (IL-10) and leukotriene B_4 (LTB_4) were not detectable. All three glucocorticosteroids (budesonide, fluticasone propionate and prednisolone) completely inhibited the production of both eicosanoids and cytokines. The production of eicosanoids was more sensitive to these glucocorticoids than the production of cytokines. The observed differences in the kinetics of the production of eicosanoids and cytokines stress the importance of time course experiments in studies on the effect of drugs on mononuclear cells.

Key words: Glucocorticosteroids, Cytokines, Arachidonic acid metabolites, U937

Introduction

Inflammation of the airways underlies a major part of the clinical symptoms in asthma and chronic obstructive pulmonary diseases (COPD). A number of studies support the involvement of mononuclear phagocytes in asthma.[1–3] Alveolar macrophages phagocytose inhaled particles and antigens, but as inflammatory cells they are known to produce potent inflammatory mediators, including proteins and lipids mediators, which are able to influence other cell types.

Several features of asthma can be mimicked *in vitro* and *in vivo* by these secretory products.[4] We and others have previously shown that prostaglandin D_2, prostaglandin $F_{2\alpha}$ and a stable analogue of thromboxane A_2 all constrict human airway muscle.[5] Leukotriene B_4 (LTB_4) increases vascular permeability and enhances adherence and migration of granulocytes. Inhalation of LTB_4 results in peripheral neutropenia and the accumulation of leucocytes in the airway wall.[6] Leukotriene C_4 (LTC_4) induces bronchoconstriction, reduces the clearance of mucus and increases vascular permeability.[7] Cytokines, such as IL-1β, IL-6 and TNF-α, play an important role in immune reactions and regulate the growth and state of activity of many cells in inflammation including the airway inflammation present in asthma.[8] Both eicosanoids and cytokines can also be released from inflammatory cells in culture. Some studies have investigated the regulation of cytokines and eicosanoids in macrophage cell lines or macrophages stimulated with various inflammatory agents.[9–13]

The human monoblastoid U937 tumour cell line is widely used as a model for the maturation of monocytic cells.[14] U937 is an established human monoblast cell line derived from the pleural exudate of a patient with diffuse histiocytic lymphoma. Maturation of U937 can be induced by incubation with different agents, including retinoic acid,[15] vitamin D derivatives,[16] cytokines[17] and phorbol esters.[18] Phorbol ester-induced maturation results in cessation of proliferation and significant alterations in the morphology of the cells. Under standard conditions, U937 cells grow in suspension and exhibit a smooth

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and round surface, but they become adherent, start to form cell clusters and extend pseudopodia upon phorbol ester treatment.[18–20] The morphological changes observed during this maturation process suggest functional changes of the U937 cells.

We studied the kinetics of the production of several cytokines, IL-1β, IL-6, IL-10, IFN-γ and TNF-α and eicosanoids PGE_2, TxB_2 and LTB_4 in U937 to clarify whether mediators are sequentially released by activated mononuclear cells. These mediators were studied, because they are thought to be of importance in the pathogenesis of the airway inflammation in lung diseases. Furthermore, the effectiveness of glucocorticosteroids to suppress the formation of inflammatory mediators was investigated. We used budesonide, fluticasone propionate and prednisolone, because they are the most important drugs currently used in the treatment of asthma.

Materials and methods

Cell culture

U937 cells (American Type Culture Collection USA, batch 1593.2) were cultured in RPMI 1640 medium (Gibco, UK) supplemented with penicillin (ICN, USA; 100 μg ml^{-1}), streptomycin (ICN, USA; 100 μg ml^{-1}), glutamax-I (Gibco, UK; 10 mM) and 10% foetal bovine serum (FBS; Gibco, UK) in tissue culture flasks (Costar, UK) at 37°C humidified atmosphere with 5% carbon dioxide.

Maturation of U937 cells was induced with 250 ng ml^{-1} PMA (Sigma, USA). Cells were cultured for 24 h with PMA and further grown for 48 h in fresh complete medium. Cell viability was assessed by trypan-blue exclusion in the non-adherent untreated U937 as well as in the PMA treated U937 cells. The viability of the cells was >95%.

The adherent cells were scraped from the tissue culture flasks with a rubber policeman and aliquots of 0.5×10^6 cells in 1 ml were put in 24 wells plastic multiwell culture plates (Costar, UK) and incubated with 1 μg ml^{-1} or 5 μg ml^{-1} or without LPS from *Escherichia coli* (Serotype 0111:B4; Sigma, USA). The cells were incubated for 1, 3, 6, 8, 10, 12, 24, 48 and 96 h in separate wells at 37°C. Cell-free supernatants were collected and stored at –80°C until measurements of lipid mediators and cytokines.

Incubation with steroids

To ensure that the U937 cell line in our investigations could be used to evaluate glucocorticoid responsiveness, we performed the experiment with steroids. We used three corticosteroids, namely budesonide (Sigma, USA), fluticasone propionate (gift of Glaxo Wellcome, UK) and prednisolone sodium phosphate (Genfarma, NL). The drugs were used in the final concentrations: 10^{-11}, 10^{-9} and 10^{-7} M.

The cells were pre-treated with PMA, the drugs were added to the cell cultures 24 h prior to stimulation with LPS. From this set point [t = 0] the cells were incubated for 1, 3, 6, 8, 10, 12, 24, 48 and 96 h in the presence or absence of the drug and/or LPS. The cell-free supernatants were stored at –80°C until measurements.

Cell viability was not altered after drug treatment.

Measurements of cytokines

IL-1β (R&D systems, USA), IL-6 (CLB, NL), IL-10 (Pharming, USA), TNF-α (Pharming, USA) and IFN-γ (Medgenix, Belgium) levels were measured using enzyme-linked immunosorbent assay (ELISA).

Measurements of eicosanoids

The supernatants were processed as described in detail previously with slight modifications.[21] SepPak C_{18} cartridges (Waters Ass., USA) were activated with 10 ml of methanol pre-washed with 10 ml distilled water. Two hundred and fifty μl of supernatant was applied to the columns and rinsed with 2.5 ml methanol, dried with a Savant Speed-Vac concentrator, and dissolved in 1 ml radioimmunoassay (RIA) buffer. PGE_2 and TxB_2 production were measured by RIA (antibodies were obtained from PerSeptive Diagnostics, USA, tritiated antigens from Amersham, UK and standards from Sigma, USA). LTB_4 levels were measured by enzyme immuno assay (EIA) kits, which were obtained from Amersham, USA.

Statistical analysis

Each drug was studied in four separate experiments and the values are expressed as mean ± s.e. mean. Statistical comparisons between control and drug-treated cell cultures were made by ANOVA followed by paired *t*-test. A $p<0.05$ was considered significant.

Results

LPS-induced cytokine production after pre-treatment with PMA

In standard medium without PMA, U937 cells had a smooth and round surface and did not produce the cytokines and eicosanoids studied (data not shown). After addition of PMA they became adherent by forming cell clusters, extended pseudopodia and, as shown in Fig. 1, released IL-1β, IL-6 and TNF-α in response to LPS stimulation. However, LPS did not induce the PMA-treated cells to produce INF-γ or IL-10 (data not shown). After incubation with LPS the amounts of IL-1β increased significantly at 48 and 96 h, which seemed to be concentration dependent. IL-6 levels already increased significantly after 6 h, after which the levels reached a plateau. TNF-α production

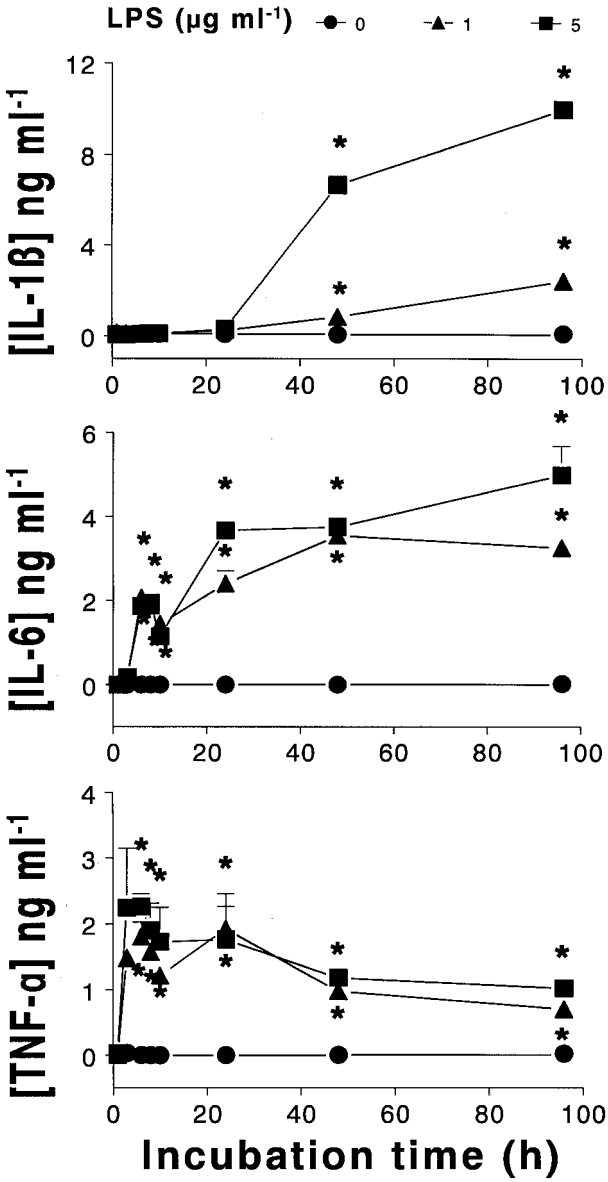

FIG. 1. Kinetics of IL-1β (*top panel*), IL-6 (*middle panel*) and TNF-α (*lower panel*) production in PMA-treated U937 cells which were stimulated with LPS. Data represent mean ± s.e. mean obtained from four separate experiments. *$p<0.05$ versus response without LPS. When no error bar is visible, error falls within the limit of the symbol.

showed a different pattern compared with IL-1β and IL-6; its levels increased after 6 h of incubation with LPS, but started to decrease after 24 h.

LPS-induced eicosanoid production after pre-treatment with PMA

We determined the effects of LPS on PGE_2, TxB_2 and LTB_4 production by U937 cells which were pre-treated with PMA. Without PMA, U937 cells did not produce any of the eicosanoids studied (data not shown). The cells primed with PMA released PGE_2 and TxB_2, but not LTB_4, in response to LPS (Fig. 2). This effect was time-related. The levels of TxB_2 already reached a plateau phase after 6 h, whilst the levels of PGE_2 increased slowly with time.

Effect of glucocorticosteroids on the LPS-induced production of inflammatory mediators by PMA pre-treated U937 cells

Glucocorticosteroids downregulate the cytokine expression of human alveolar macrophages.[22] To investigate whether the U937 cells could be used as a model to study glucocorticoid responsiveness in monocytes/macrophages, we incubated the cells with budesonide, fluticasone propionate or prednisolone. Figures 3, 4 and 5 show the data obtained

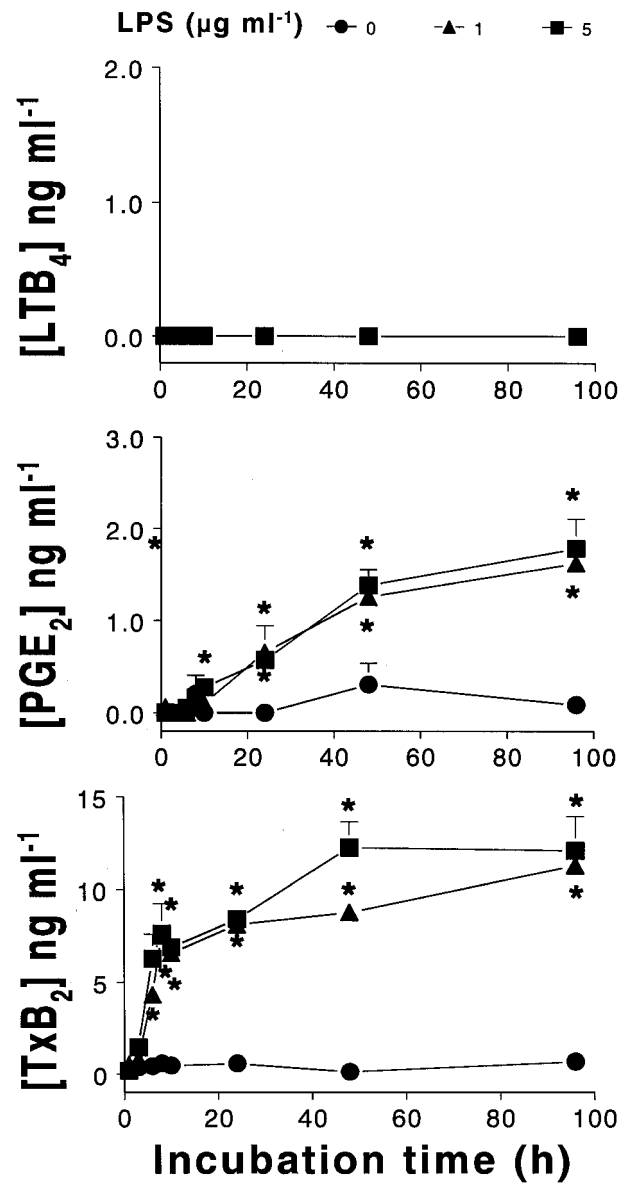

FIG. 2. Kinetics of LTB_4 (*upper panel*), PGE_2 (*middle panel*) and TxB_2 (*lower panel*) production in PMA-treated U937 cells which were stimulated with LPS. Data represent mean ± s.e. mean obtained from four separate experiments. *$p<0.05$ versus response without LPS. When no error bar is visible, error falls within the limit of the symbol.

with budesonide, fluticasone proprionate and prednisolone respectively. The production of IL-1β and IL-6 was dose-dependently inhibited by all three steroids. At 10^{-7} M concentration, all three steroids completely abolished the production of IL- 1β, IL-6 and TNF-α induced by LPS. In the absence of LPS no production could be found in the supernatant (data not shown). Similarly, the production of the eicosanoids (PGE_2 and TxB_2) was already inhibited by all three glucocorticosteroids at concentrations of 10^{-11} M.

Discussion

In the present study we have shown that PMA induced morphological differentiation of U937 cells. We also found that PMA pre-treated U937 cells needed LPS for the production of both cytokines (IL-1β, IL-6 and TNF-α and eicosanoids (PGE_2 and TxB_2). IL-6 was already detectable after 6 h of incubation, whereas IL-1β could be detected after 48 h. The production of both cytokines reached a plateau, in contrast to the transient induction of TNF-α which was maximal between 6 and 24 h. Furthermore, we showed that the production of eicosanoids could be inhibited by lower concentrations of glucocorticoids as compared with the production of cytokines.

Unstimulated U937 cells exhibited a smooth and round surface, but after PMA addition they became adherent by forming cell clusters and extended pseudopodia, which is in agreement with the findings of Hosoya & Marunouchi.[20] Unlike these changes in morphology, the production of cytokines and eicosanoids could be induced by LPS and PMA together only. However, Hass *et al.*[23] found that U937 cells released IL-1β and TNF-α after TPA treatment without LPS. IL-1α and IL-6 could not be detected in their system. The basis of these differences is unclear. A

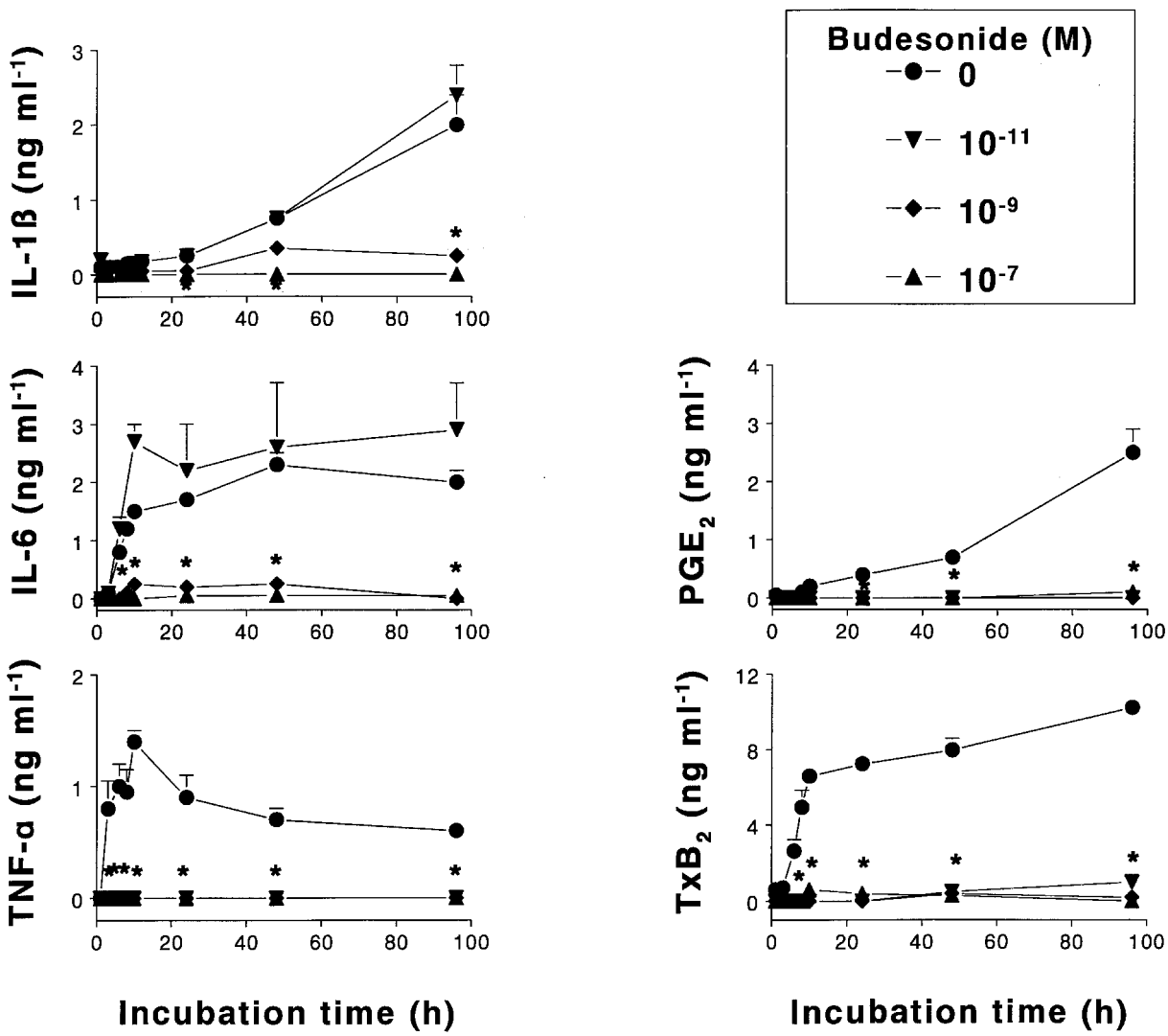

FIG. 3. Effects of budesonide on IL-1β, IL-6, TNF-α, PGE_2 and TxB_2 production by U937 cells pre-treated and stimulated with PMA and LPS respectively. Data represent mean ± s.e. mean obtained from four separate experiments. *$p<0.05$ compared with control cultures. When no error bar is visible, error falls within the limit of the symbol.

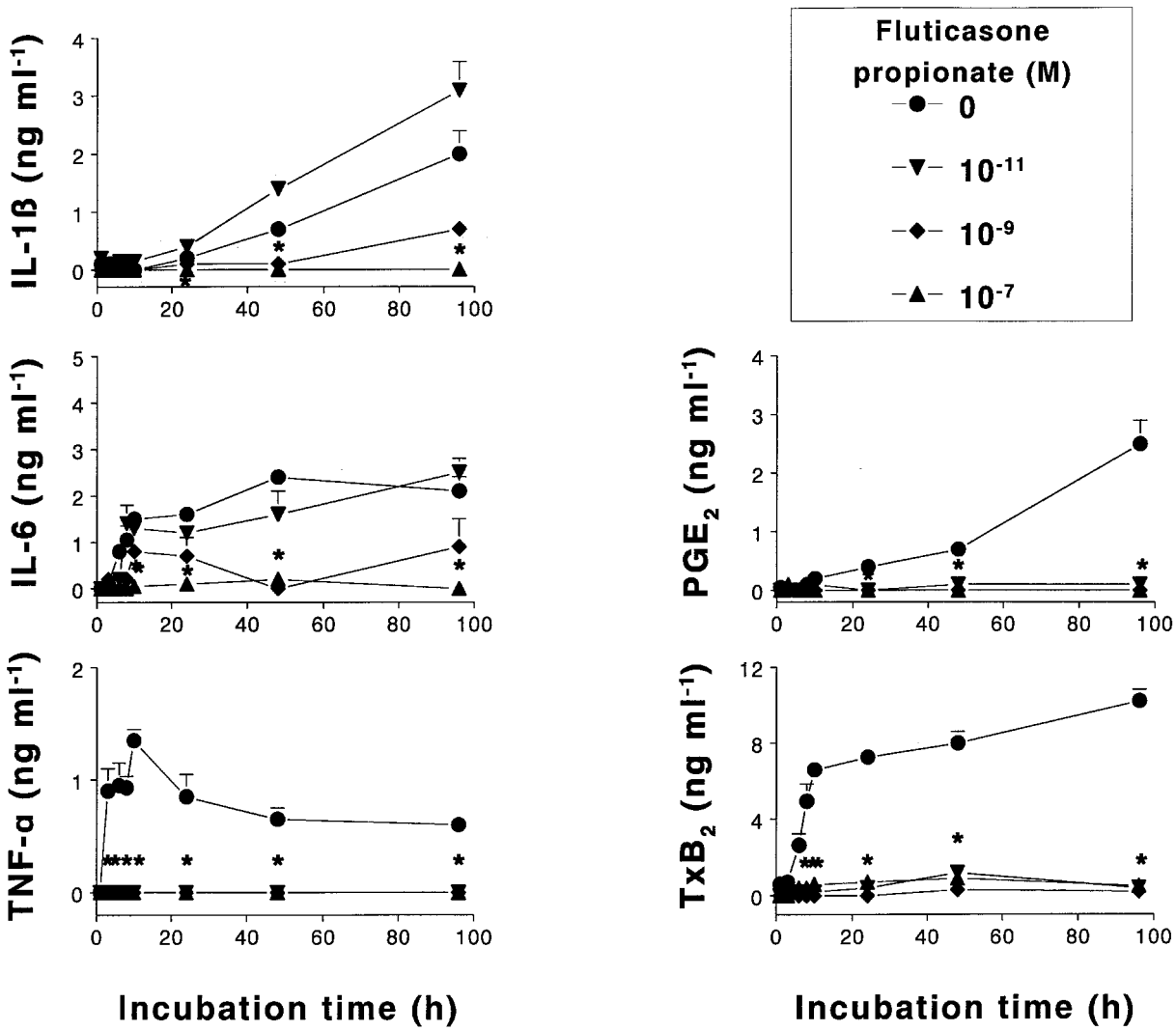

FIG. 4. Effects of fluticasone propionate on IL-1β, IL-6, TNF-α, PGE_2 and TxB_2 production by U937 cells pre-treated and stimulated with PMA and LPS respectively. Data represent mean ± s.e. mean obtained from four separate experiments. *$p<0.05$ compared with control cultures. When no error bar is visible, error falls within the limit of the symbol.

possible explanation may be that a subclone of U937 was used different from the one used in our experiments. However, Hass *et al.*[23] also showed in their system that TNF-α was expressed earlier (already after 2–4 h) than IL-1β (after 24–48 h), and this difference in kinetics is in agreement with our results.

In this investigation we showed an increase in TNF-α production until 12 h of incubation with LPS. TNF-α is known as a macrophage activator.[12] Therefore, it can be expected that after 12 h all the U937 cells present in the well would be activated and thus the production of TNF-α will be terminated. Taimi *et al.*[24] observed that IL-6 is produced by PMA-differentiated U937 cells after stimulation with LPS. We showed that the production increased significantly after 6 h of incubation, after which the levels reached a plateau. This may indicate that at this point the cells have stopped producing IL-6 and that the amount of IL-6 is the actual amount that the cells have produced without any breakdown of IL-6. This plateau could also have been reached because the rate of breakdown of IL-6 equalled the production of IL-6. We demonstrated that the amount of IL-1β secreted by PMA pre-treated U937 gradually increased after incubation with LPS even after 48 and 96 h. Previous studies have shown that IL-1 generation is dependent on the stage of differentiation.[25] The basis of the differential and sequential expression of the three cytokines studied here may be caused by sequential secretion or gene expression during PMA/LPS-induced differentiation.

We observed that PMA-treated U937 produced the eicosanoids PGE_2 and TxB_2, but no LTB_4, after incubation with LPS. Since LTB_4 was not detectable, the generation of leukotrienes appears to represent a property of cells at a later state in the differentiation along the monocyte/macrophage lineage. Köhler found that U937 secreted PGE_2 and TxB_2, but no LTB_4

after incubation with TPA and arachidonic acid for 1–24 h.[26] It is known that human peritoneal macrophages do generate lipoxygenase products after incubation with Ca-ionophore A23187.[13] However, in this study we found no effect of A23187 (1 and 5 μM for 15 min) on U937 (data not shown).

Previous studies have reported the presence of glucocorticoid receptors in U937 cells.[27] In a recent study we have demonstrated under our culture conditions, using the unadapted receptor assay, specific binding of ^{3}H-labelled dexamethasone to these cells. U937 cells appeared to have 17.1 +/– 5.6 × 10^3 sites per cell and a K_d of 5.3 +/– 1.0 nM.[28] These findings suggested that U937 cells are glucocorticoid responsive. Knudsen has demonstrated that glucocorticosteroids downregulate the IL-1β gene expression by U937,[29] which has been confirmed in human alveolar macrophages recently.[22] In this study we confirmed the glucocorticoid-induced downregulation of IL-1β in U937 cells, and additionally we showed that also the induction of IL-6 and TNF-α is inhibited by different classes of glucocorticoids. Interestingly, glucocorticoids were able to inhibit the induction of both PGE_2 and TxB_2 at a much lower concentration (10^{-11}M) as compared with the inhibition of the cytokines studied (10^{-7} M). This difference may be explained by differences in the underlying working mechanism of glucocorticoids. Glucocorticoids are thought to interfere with the production of eicosanoids via the induction of lipocortins. Lipocortin-1 can be induced in differentiated U937 cells by glucocorticoids.[30] However, the downregulation of cytokine expression by glucocorticoids is thought to occur at the level of either transcription or translation; glucocorticoids may interact with negative glucocorticoid response elements in the gene of some cytokines, may interact with other transcription factors or may decrease the

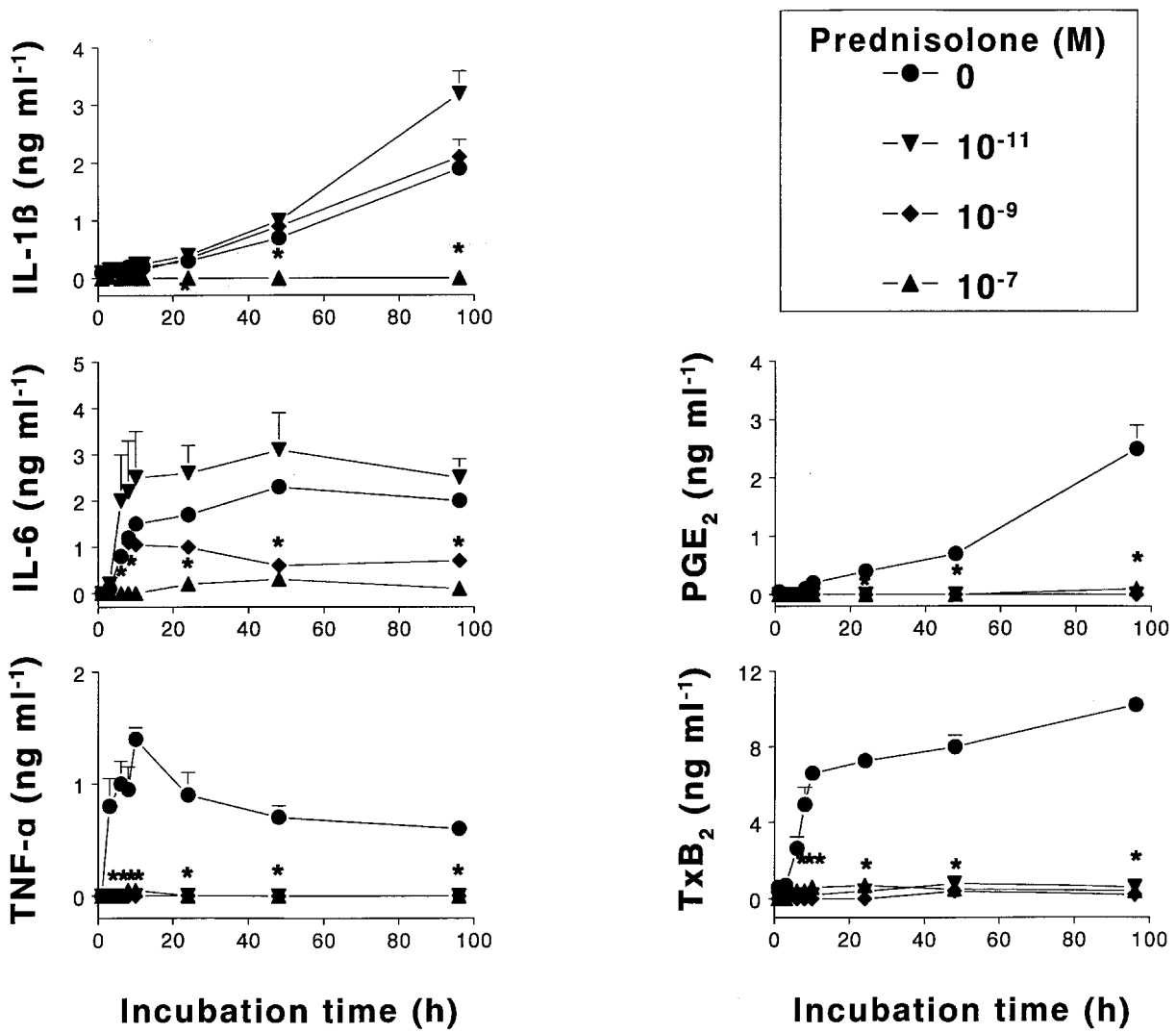

FIG. 5. Effects of prednisolone on IL-1β, IL-6, TNF-α, PGE_2 and TxB_2 production by U937 cells pre-treated and stimulated with PMA and LPS respectively. Data represent mean ± s.e. mean obtained from four separate experiments. *p<0.05 compared with control cultures. When no error bar is visible, error falls within the limit of the symbol.

stability of mRNA.[31] Future studies in U937 should focus on the question, which of these three mechanisms, or combination of mechanisms, underlies the glucocorticoid effects on the cytokine expression observed here.

In conclusion, the results of this study show that U937 cells can be used as a model to study the production of selected inflammatory mediators that are believed to be important in the pathogenesis of asthma. Additionally, U937 cells can be used to study the effects of glucocorticoids on these mediators. The observed differences in the kinetics of the production of eicosanoids and cytokines stress the importance of time course experiments in studies on the effects of drugs on mononuclear phagocytes.

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